Brain & Development 41 (2019) 567–576

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

The developmental expression of metabotropic glutamate receptor 4 in prenatal human frontal lobe and neurogenesis regions

Yan Xiang [c], Hua Han [a], Shengfeng Ji [a], Liang Wei [b], Pengbo Yang [a,*], Junfeng Zhang [b,*]

[a] Department of Human Anatomy, Histology and Embryology, Xi'an Jiaotong University Health Science Center, China
[b] Department of Human Anatomy & Shaanxi Key Laboratory of Brain Disorders, Xi'an Medical University, China
[c] Department of Internal Medicine, Xi'an Huashan Central Hospital, China

Received 17 November 2018; received in revised form 22 February 2019; accepted 25 March 2019

Abstract

Backgrounds: Metabotropic glutamate receptors, besides ionotropic receptors, mediate the complicated effect of glutamate on neurogenesis. Previous studies showed that metabotropic glutamate receptor 4 (mGluR4) regulated the proliferation and differentiation of neural stem/progenitor cells in vitro. However, little is known about the expression pattern of mGluR4 on prenatal central nervous system in vivo, especially the human being.

Methods: The normal brain tissues of human fetus were collected and divided into 4 groups according to the gestational age: 9–11 W, 14–16 W, 22–24 W and 32–36 W. Then the expression of mGluR4 was evaluated at mRNA and protein levels by means of PCR or immunohistochemistry method, respectively. The type of cell expressing mGluR4 was further investigated using double-labeling immunofluorescence.

Results: RT-PCR showed that the mRNA of mGluR4 could be detected in frontal lobe from 9 W to 32 W and real-time PCR quantificationally demonstrated the mRNA increased with development. Similarly, immnoreactivity was found in all layers of frontal lobe, VZ/SVZ. The intensity scores analysis showed that the staining became stronger and the range extended gradually with development. The double-labeling immunofluorescence showed that mGluR4 was present in neural stem/progenitor cells (nestin-positive cells after 9 W), young neurons (DCX-positive cells after 9 W), mature neurons (NeuN-positive cells in cortex after 32 W), as well as typical astrocytes (GFAP-positive cells in medulla after 32 W).

Conclusion: These results supply an important evidence that mGluR4 is expressed in prenatal human cerebrum, and main kinds of cells related to neurogenesis are involved in its expression.

Keywords: Metabotropic glutamate receptor 4; Neurogenesis; Frontal lobe; Human fetus

1. Introduction

It is accepted that glutamate, one of extracellular signals, regulates the proliferation of neural stem cells (NSCs) or neural progenitor cells (NPCs) and makes them differentiating into a specific neuronal phenotype during development of nervous system. Glutamate regulates the neurogenesis on diverse and elaborate manner, via its rich receptors including ionotropic and metabotropic glutamate receptors [1,2]. Early studies showed that glutamate could reversibly depress the proliferation of NPCs in embryonic cortical explants through AMPA/kainate receptors [3]. In addition, acute NMDA receptor blockade increased the number of proliferating

* Corresponding authors.
E-mail addresses: yangpengbo74@126.com (P. Yang), zhangjf_08@126.com (J. Zhang).

https://doi.org/10.1016/j.braindev.2019.03.010
0387-7604/

granule cell precursors and newborn neurons in the dentate gyrus [4,5].

Nowadays, it is well known that there are eight subtypes of metabotropic glutamate receptors (mGluRs), named from mGluR1 to mGluR8. Briefly, mGluR1 and mGluR5 belong to the group I mGluRs; group II consists of mGluR2, mGluR3; and the group III includes mGluR4, −6, −7, and −8 [6–8]. With the identification of mGluRs subtypes, a growing body of research focused on the role of mGluRs in neurogenesis recently.

mGluR5 of the group I mGluRs could be expressed by neural stem/progenitor cells in pre- or postnatal brain in rodents and human beings [9–13]. Activation of mGluR5 increases the proliferation of neural stem cells and increases the survival of neural stem/progenitor cells that were going to differentiate into neurons [14,15]. Injection of mGluR5 agonist into brain facilitates the neurogenesis in rat brain hemorrhage [16]. mGluR3 of the group II mGluRs is expressed by NPCs of the developing and adult rodent forebrain where the activation of mGluR3 supports cell survival and proliferation [17–19]. On NPCs isolated from the SVZ of perinatal rat brain, the activation of group II mGluRs increases cell proliferation while the mGluR3 agonist NAAG supports cell survival [17].

The role of the group III mGluRs is even less clear and conflicting results have been reported. Activation of group III mGluR was found to reduce cell proliferation, decrease neuronal differentiation, but increase astrocytic differentiation of mouse or human NPCs, and those effects could be reversed by the group III mGluR antagonist CPPG [19]. Treatment with a specific enhancer of mGluR4 (PHCCC) reduced proliferation and promoted differentiation of cerebellar granule cell neuroprecursors [20]. However, others found that activation of group III metabotropic glutamate receptor (mGluR7 or mGluR4) promoted the proliferation and differentiation of NPCs with changes in phosphorylation of MAPK signaling pathways [21,22].

In order to prove the functional importance of neurotransmitter receptor during development, indispensable evidence usually comes from the examination of its mRNA or protein expression. In contrast to the functional studies, very few investigations have been focused on the developmental pattern of mGluR4 and its cellular localization on the neural stem/progenitor cells. Nakamichi ever identified the mRNA of mGluR4 in freshly isolated nestin-positive (nestin is a marker of NPCs) cortical progenitors from prenatal mice [19]. Furthermore, functional mGluR4 had been found to be expressed in embryonic stem cell-derived neural stem/progenitor cells and cerebellar granule cell neuroprecursors [20,23,24]. However, information regarding the developmental distribution and specific cellular localization of mGluR4 in human brain remains to be elucidated. Here we focused on the distribution of mGluR4 on the prenatal frontal lobe, particularly the active zones of neurogenesis such as ventricular zone (VZ) and subventricular zone (SVZ). Then the cellular localization of mGluR4 on the main cells related to neurogenesis was explored with double immunofluorescence.

2. Materials and methods

2.1. Human brain samples

Final samples were 34 normal fetuses at various gestational weeks coming from elective, spontaneous or medically induced abortions. Detailed information about the cases was seen in our previous study [25]. The whole protocol received an approval from the Ethics Broad of the Xian Jiaotong University Health Science Center (No.2012-225) and followed the guidelines of the Declaration of Helsinki. Gestational age was estimated according to the last menstrual period of pregnant woman, as well as the foot length and the crown-rump length. Then the samples were divided into 4 groups: 9–11 W (n = 9), 14–16 W (n = 10), 22–24 W (n = 9) and 32–36 W (n = 6) according to the gestational age. Normal adult cerebellum (positive control for mGluR4 mRNA) was obtained at autopsy from 4 controls. Tissue was obtained usually less than 12 h after fetus death.

2.2. Tissue preparation

After abortion, the brains were rapidly removed and dissected. Small blocks of tissue from the right frontal lobe and VZ/SVZ were immediately fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 °C for about 3–7 days, then transferred to 30% sucrose in 0.1 M PB and stored until the blocks sank to the bottom at 4 °C. Coronal sections (20-μm thick) were obtained with a cryostat (Slee Technik, Mainz, Germany) and the sections were serially mounted on glutin-coated slides. At the same time, about 50 mg of tissue from the left frontal lobe and VZ/SVZ was immediately taken and immersed in liquid nitrogen for mRNA expression analysis.

2.3. mRNA expression analysis by PCR

Total RNA was extracted using TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. First, brain tissue was pulverized in 0.75 ml of TRIZOL LS Reagent using a homogenizer (Polytron Kinematica, Lucerne, Switzerland). Homogenized tissue was incubated for 5 min at room temperature, and treated with 0.2 ml of chloroform. Sample tubes were securely capped, shaken vigorously for 15 s, incubated at room temperature for

15 min, and centrifuged (12,000*g* for 15 min at 4 °C). Then the aqueous phase which included almost all RNA was transferred to a clean tube, mixed with 0.5 ml of isopropyl alcohol, incubated at room temperature for 10 min, and centrifuged (12,000*g* for 10 min at 4 °C). The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol. After centrifuging (7,500*g* for 5 min at 4 °C), RNA pellet was air dried for 10 min, then dissolved in 30 μl RNase-free water, incubated for 10 min at 55 °C, finally stored and evaluated on quality and quantity. Secondly, the cDNA was obtained using the PrimeScript TM RT reagent Kit (Takara Bio Inc., Shiga, Japan) and the starting template was 500 ng of RNA. The mixture was incubated at 37 °C for 15 min, and reverse transcription was inactivated by heating (85 °C for 5 sec). After the amplification, the PCR products were separated on 1.2% agarose gel electrophoresis. The bands were then visualized under UV light after staining by ethidium bromide.

For the real-time PCR experiments, the assay was conducted using SYBR Premix Ex Taq TM II (Takara) and ß-actin was used as the reference gene. The sequences of ß-actin and mGluR4 primers were synthesized by TaKaRa Biotechnology, and listed in Table 1. All reactions were performed in triplicate, had 20-μl reaction volumes, including 2 μl of cDNA, 10 μl of SYBR Premix EX Taq TM II, 0.8 μl each of Forward and Reverse Primer (10 μM for each primer), and 6.4 μl of ddH2O. Those were carried out using an iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Cycle threshold values were obtained from the Bio-Rad iQ5 optical System software (Bio-Rad). The reaction conditions were 95 °C for 30 s, 40 cycles of 95 °C for 3 s, 62 °C for 30 s, and finally 4 °C. The data were analyzed using the ΔΔCt method. The mGluR4 mRNA of different time points was normalized to the β-actin level and it is indicated as mean ± standard error (SE). The relative changes of mGluR4 mRNA were obtained compared to the 9 W.

2.4. Immunohistochemical study

Staining for mGluR4 was performed normally. Briefly tissue sections were treated with 0.3% H_2O_2 for 15 min, then with 2% normal goat serum (NGS) in 0.01 M PBS for 30 min, and with primary antibodies (Table 2) in 0.01 M PBS overnight at 4 °C. Then slices were incubated with secondary anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Slices were incubated with ABC Elite solution (Vector Labs), then, reacted with a solution of 0.012% H_2O_2 and 0.05% 3, 3-diaminobenzidine (Sigma, St. Louis, MO) in Tris buffer for 5 min. After every step except the treatment with NGS, slices were fully washed thrice by 0.01 M PBS. Lastly slices were dehydrated, clarified, covered by coverslips with a xylene-based mounting medium (DPX), and viewed carefully under microscopy. In the negative control, the only change of process was that primary antibodies were replaced by 0.01 M PBS. Photomicrographs were taken under an Olympus BX51 microscope equipped with a Spot camera (DP71).

Table 2
Primary antibodies used in study.

Antibody	Host	Company	Concentration
mGluR4	Rabbit	Santa Cruz	1:50
	Rabbit	abcam	1:500
Nestin	Mouse	Chemicon	1:200
DCX	Goat	Santa Cruz	1:100
GFAP	Mouse	Chemicon	1:500
NeuN	Mouse	Chemicon	1:300

2.5. Double-labeling immunofluorescence

Double-labeling immunofluorescence was performed to examine the co-localization of mGluR4 with neural stem/progenitor cells markers (nestin), immature neuron marker (DCX), mature neuron marker (NeuN) and astrocyte marker (GFAP). Tissue slice was treated with 5% bovine serum albumin for 30 min at room temperature, and then incubated overnight with primary antibodies (Table 2) at 4 °C. After washing, it was incubated for 4–6 h at room temperature with the mixture of fluorescein isothiocyanate (FITC)-conjugated and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-IgG (1:200). Finally, nucleus was stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min. After fully washing, slice was covered with Antifade Mounting Medium (Byeotime, China), and examined under an Olympus BX51 (Olympus Corporation, Tokyo, Japan) fluorescence microscope. Section

Table 1
Sequences and annealing temperatures of the primers.

Target gene	Primers	Sequences	Length (bp)	Tm (°C)	Products (bp)
mGluR4	Forward	5′-AACATCTGGTTTGCCGAGTTCTG-3′	23	62	165
	Reverse	5′-CACGGCATCGATCACAAACTG-3′	21		
ß-actin	Forward	5′-TGGCACCCAGCACAATGAA-3′	19	62	186
	Reverse	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′	25		

incubated not by primary antibody but 0.01 M PBS was taken as negative control.

2.6. Scoring of immunostaining sections and immunofluorescence-positive cell counting

The stained tissue sections were viewed and scored semiquantitatively for intensity and frequency scores. The intensity scores were evaluated for the general level of positive expression using a scale of 0–3 (0, no; 1, weak; 2, moderate; 3, strong staining) and it represents an average intensity of positive cells staining in each section. While frequency scores were evaluated as the proportion of positive expression assigning from 0 to 4 (0, <1%; 1, 2–25%; 2, 26–50%; 3, 51–75%; 4, >75%) and it represents the relative number of positive cells in the section. These two scores were then added together to form a total immunoreactivity score (IR). The number of the immunofluorescence-positive cells was counted according to the different markers, respectively. Four stained sections at the regular interval of one sample were chosen and independently evaluated by two investigators under an Olympus BX51 microscope equipped with a Spot camera (DP71) at ×200 magnifications and then took an average of 6 high-power non-overlapping fields in each section to minimize variability. These investigators were blinded to the groups of these tissue sections.

2.7. Statistical analysis

The data were analyzed by one-way ANOVA followed by Student–Newman–Keuls post hoc multiple comparisons or *t*-test (only compare between two groups) using the SPSS 13.0 software package. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Developmental expression of mGluR4 mRNA revealed by PCR in human frontal cortex

The gel electrophoresis finally showed a clear band of mGluR4 mRNA in all brain areas examined in this study (Fig. 1A). Real-time PCR results indicated that the mRNA of mGluR4 increased gradually in frontal cortex from gestational 9 W to 32 W ($p < 0.05$). However, there is no significant increase of mGluR4 mRNA between 32 W and 22 W in VZ/SVZ ($p > 0.05$). Further analysis showed that there was a significant difference in mGluR4 expression between frontal cortex and VZ/SVZ in 32 W ($p < 0.05$), not in 22 W ($p > 0.05$). Noted that the expression level of mGluR4 in frontal cortex at 32 W was far lower than the adult human cerebellar cortex where mGluR4 was highly expressed ($p < 0.05$), (Fig. 1B).

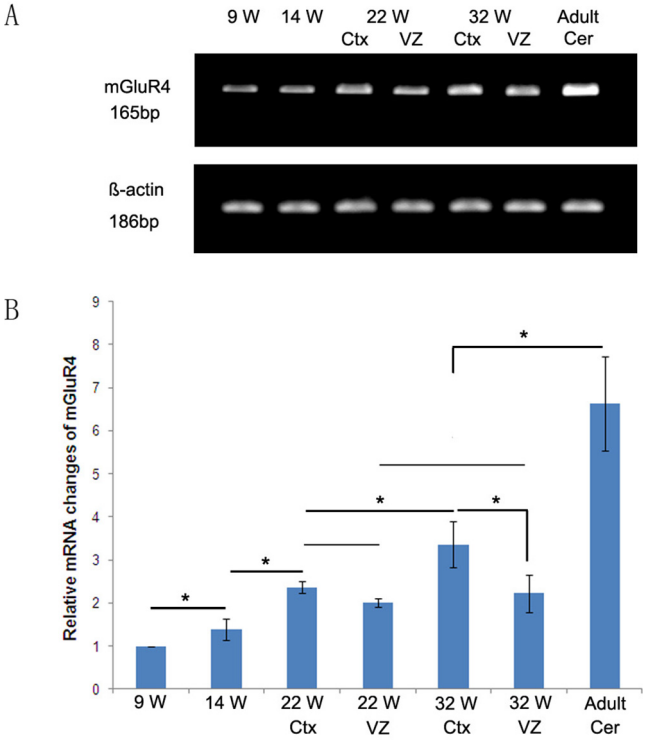

Fig. 1. Developmental expression of mGluR4 on mRNA level using PCR. The clear band revealed by RT-PCR showed that mRNA of mGluR4 was detected in the positive group (Adult Cerebellum) as well as all developmental groups (A). Real-time PCR results quantificationally implied it increased gradually in frontal cortex from gestational 9 W to 32 W. However, the mRNA expression level of mGluR4 was lower in VZ than cerebral cortex at 32 W (B). Values are mean ± SE, *$P < 0.05$ and $n = 28$. Ctx, cortex; VZ, ventricular zone; Cer, cerebellum.

3.2. Distribution of immuno-positive cells for mGluR4 in human frontal cortex and VZ/SVZ

Moderate immunoreactivity for mGluR4 was found in marginal zone (MZ), cortical plate (CP) and the innermost part of ventricular zone (VZ) about 9 W. The weak immuno-positive cells were also detected in intermediate zone (IZ) and subventricular zone (SVZ). The positive cells in MZ and VZ appeared a small, clear, round or oval cell body and they were arranged tightly into one or more layers. However, the positive cells scattered in CP and were slightly bigger than MZ and VZ (Fig. 2 A, B).

At 14 W, the distribution profile of positive cells was very similar to 9 W. Nevertheless, the extent of immnoreactivity, and the cell bodies in SVZ and IZ increased gradually (Fig. 2 C-E; Table 3).

With the marked thickening of the frontal cortex, the positive cells were diffusely located in all layers at 22 W.

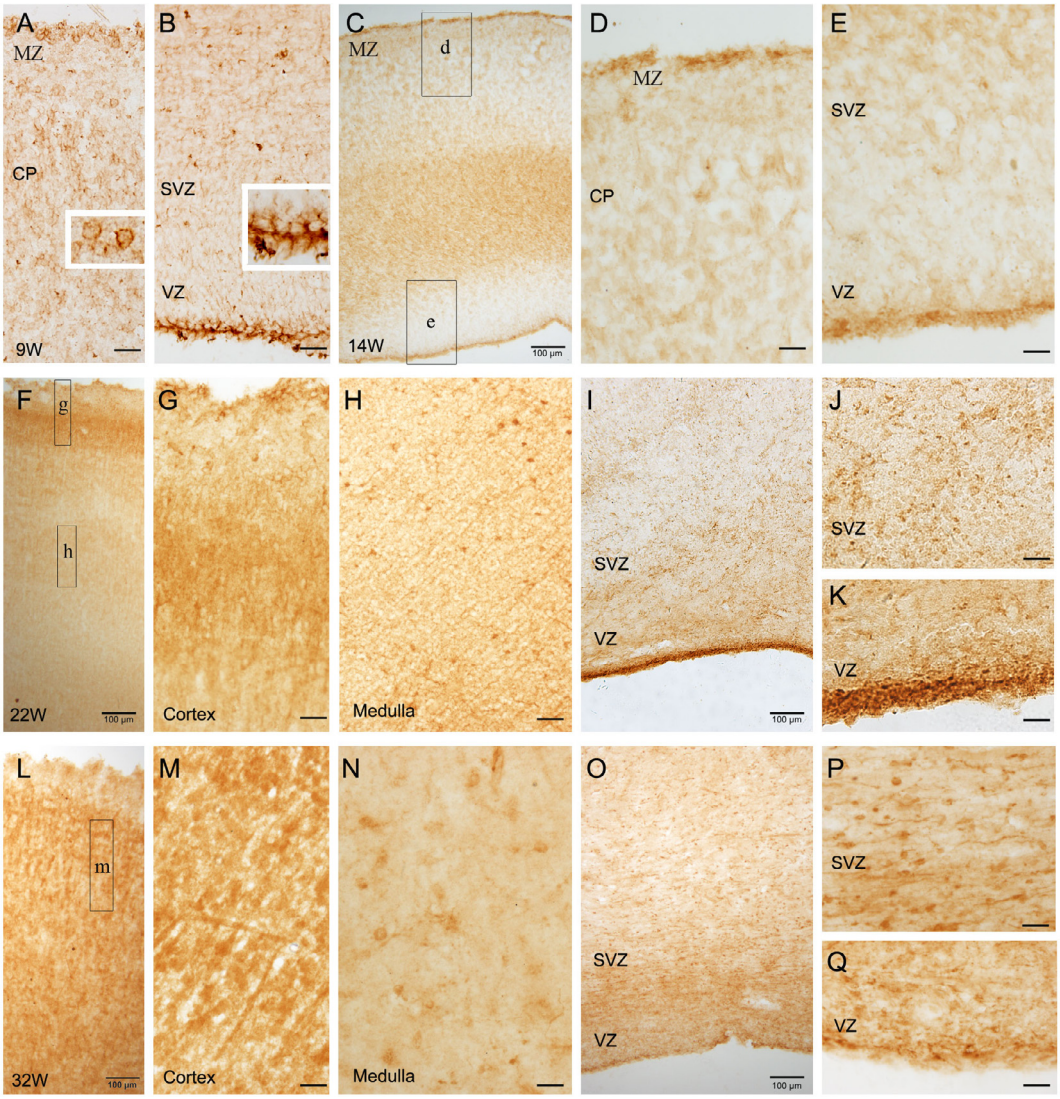

Fig. 2. Developmental profile of mGluR4 protein using immunohistochemistry staining in fetal frontal lobe. Marked staining was mainly presented in MZ and VZ at 9 W. The insets in A and B were distinct immuno-positive cells at high magnification in MZ and VZ, respectively (A, B). At 14 W, moderate immunoreactivity expanded to SVZ, besides MZ and VZ. Panel D and E were high magnification of the boxes labeled by letter d, e in C, respectively (C-E). Strong staining appeared in all layers of frontal cortex (F, G) and medulla (H), SVZ/ VZ (I-K) beneath the cortex at 22 W. Panel G and H were high magnification of the boxes labeled by letter g, h in F, respectively. At 32 W, the distribution pattern was similar to 22 W (L-Q), except that the staining was intensively stronger and some positive processes were seen in medulla (N) and SVZ (P). The panel M was high magnification of the boxes labeled by letter m in L. The uncharted scale bar (except insets in A and B, which was 20 μm) was 50 μm. CP, cortical plate; SVZ, subventricular zone.

The immnoreactivity became stronger than 14 W and some slight processes were found about round or oval bodies (Fig. 2 F, G; Table 3). There were many positive cells in medulla and the reactivity was found in both cell membrane and cytoplasm (Fig. 2 H). The positive cells in VZ and SVZ displayed a diversified outline and a varying extent of reactivity in cell membrane and cytoplasm. Moreover, positive cells in SVZ were more than VZ in number (Fig. 2 I-K; Table 3).

At 32 W, the strongest staining was found in cortex, VZ/SVZ as well as medulla among the four time-point groups, and the cell membrane and cytoplasm were the main parts where the immunostaining located (Fig. 2 L, O; Table 3). Many positive cells, with a round, oval or pyramidal cell bodies, were detected in cortex (Fig. 2 M) whereas the positive cells were sparse in medulla (Fig. 2 N). Meanwhile, there were many positive cells with clear axons or dendrites in VZ/SVZ (Fig. 2 O-Q).

Overall, the extent of immunoreactivity became stronger and the staining range extended gradually as the development of brain (Table 3). In addition, the immunoreactivity was preferentially located in cell membrane before 14 W whereas it extended from cell

Table 3
Scoring of mGluR4 immunoreactivity with development.

	9w	14w	22w	32w
Frontal lobe	3.00 ± 0.10	2.80 ± 0.13	4.78 ± 0.15*	7.83 ± 0.31*
VZ/SVZ	3.22 ± 0.15	3.10 ± 0.10	4.33 ± 0.17*	4.17 ± 0.17

* $p < 0.05$ compared with the left younger group.

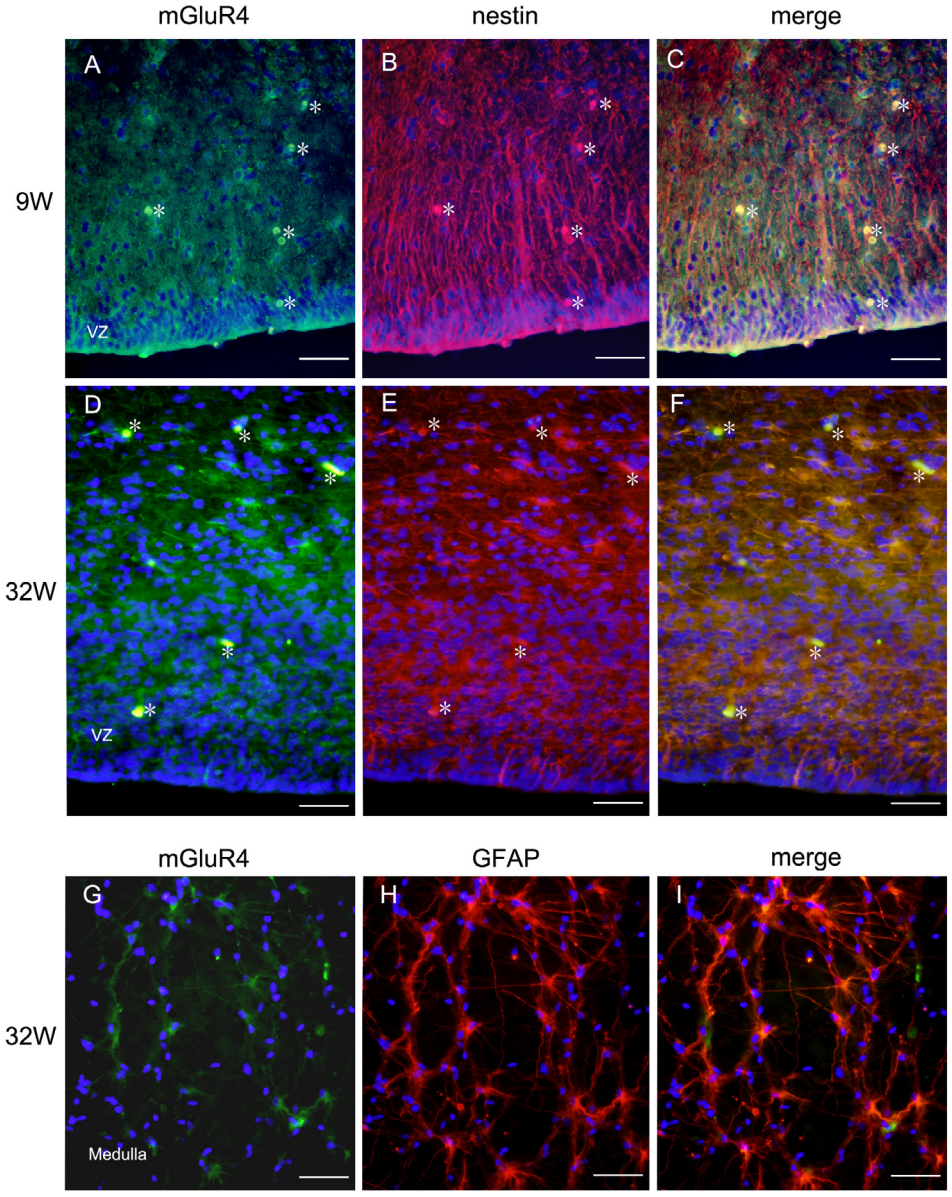

Fig. 3. Co-localization of mGluR4/nestin and mGluR4/GFAP in fetal cerebrum. Many mGluR4 (green) and nestin (red) double-labeled cells were mainly found in VZ/SVZ at 9 W (A-C) and the number of double-labeled cells markedly decreased at 32 W (D-F). However, a few mGluR4 (green) and GFAP (red) double-labeled cells in medulla appeared until 32 W (G-I). Note that the nuclei were stained blue by DAPI and the non-specific intense red or green fluorescence may be due to the microvasculature, including erythrocytes marked by asterisks in panel A-C. Scale bar was 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

membrane to cytoplasm and processes after 22 W. The positive cells grew bigger and had various morphologies gradually. Therefore, the results implied that mGluR4 could be expressed by main kinds of cell among the development of human frontal cortex.

3.3. Co-localization of mGluR4 and nestin, GFAP, DCX as well as NeuN

The secondary purpose of this study was to provide the information concerning the cellular localization of

mGluR4. The double immunofluorescence staining was used to determine the type of cells expressing mGluR4 during development. Nestin is a recognized marker to indicate the neural stem/progenitor cells. The double-labeled cells of mGluR4 and nestin could be found in all time-point groups, especially in 9 W. The primary locations of double-labeled cells were the innermost layer of VZ, and SVZ (Fig. 3 A-F). The number of double-labeled cells decreased gradually with development (Table 4).

Astrocyte (a kind of important cell of neuroglia) emerged, then increased and matured gradually in the middle and later periods of gestation. The results showed that a great body of double-labeled cells of mGluR4 and GFAP were detected in the medulla deep to the frontal cortex in 22 W and 32 W groups (Table 4). The double immunofluorescence staining was located in the cytoplasm, cell membrane and several thin processes around body (Fig. 3 G-I). However, the intensity of immunoreactivity for mGluR4 was markedly lower than GFAP in those double-labeled cells.

Neural stem/progenitor cells first differentiated into immature neurons then grew into mature neurons with development in neurogenesis. DCX-positive cells were supposed as immature, migrating neurons. The double-labeled cells of mGluR4 and DCX were mainly found in VZ and CP in 9 W. They were located in the innermost layer of VZ and MZ, and arranged into one layer on the surface of cortex (Fig. 4 A-F). Like mGluR4 and nestin double-labeled cells, they were also

Table 4
Developmental changes of number of double-labeled cells.

	9w	14w	22w	32w
mGluR4/nestin (VZ/SVZ)	1498.64 ± 6.25	1030.90 ± 13.13*	625.56 ± 12.84*	203.00 ± 3.27*
mGluR4/GFAP (medulla)			31.22 ± 1.37	79.33 ± 1.86*
mGluR4/DCX (CP and VZ/SVZ)	509.89 ± 5.65	200.50 ± 2.66*	49.89 ± 4.59*	19.50 ± 1.26*
mGluR4/NeuN (CP)			112.12 ± 1.67	389.50 ± 3.50*

* $p < 0.05$ compared with the left younger group.

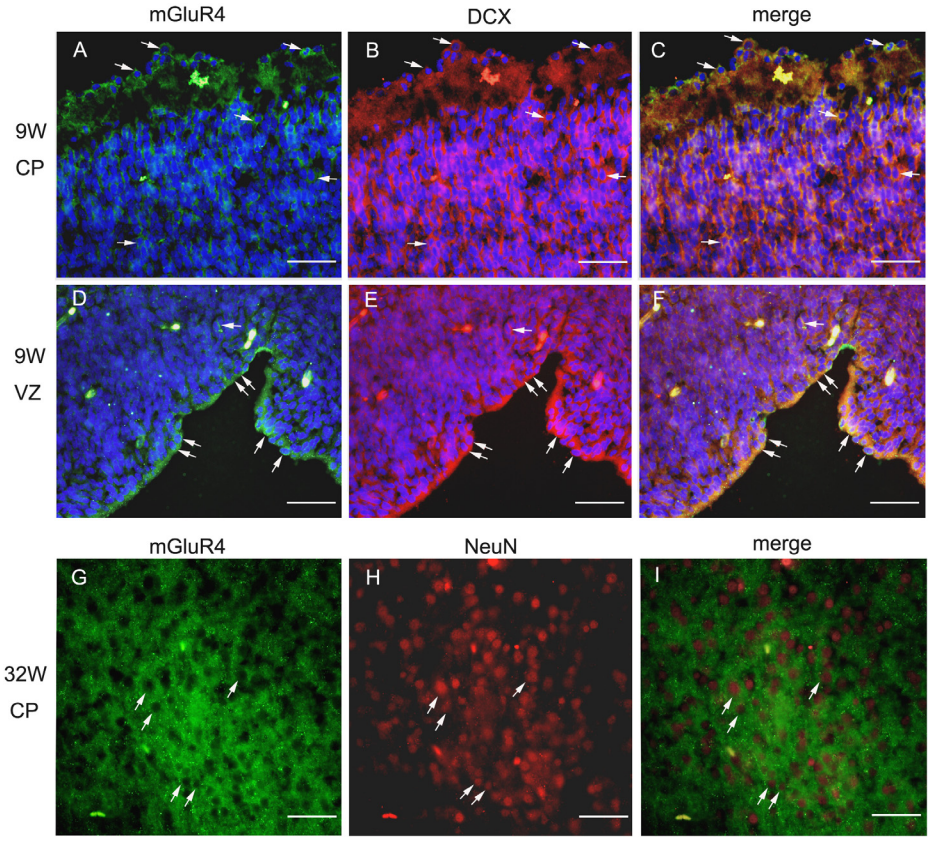

Fig. 4. Co-localization of mGluR4/DCX and mGluR4/NeuN in fetal cerebrum. Some mGluR4 (green) and DCX (red) double-labeled cells were mainly found in MZ, CP (A-C) and VZ/SVZ (D-F) at 9 W and the number of double-labeled cells sharply decreased after it. However, a few mGluR4 (green) and NeuN (red) double-labeled cells appeared in cortex at 32 W (G-I). Arrows indicated the typical double- labeled cells. Scale bar was 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased in number with development (Table 4). NeuN was usually used to mark mature neurons in neuroscience study. Results showed that there were many mGluR4 and NeuN double-labeled cells in the frontal cortex in 32 W and its number was significantly more than 22 W (Fig. 4 G-I; Table 4). Those results implied mGluR4 could express on immature and mature neurons.

4. Discussion

4.1. mGluR4 expressed in the human fetal cerebrum

Glutamate, via its diverse receptors, elaborately plays a role in the regulation of neurogenesis not only in the perinatal brain development, but also in the intrinsic repairs in adult brain after some diseases [26]. Among the glutamate receptors, mGluR4 has not been extensively focused on before. Examining the expression of mGluR4 on mRNA and protein levels during development will supply an important evidence for the functional study of mGluR4 on neurogenesis.

A growing body of evidence suggests that mGluR4 is expressed in postnatal rodent or human brain. The mRNA and protein expression for mGluR4 could be detected at birth, then increase gradually and in adult preferentially express in cerebellum, hippocampus, basal ganglia and thalamus, etc [27–31]. However, so far the data about the expression of mGluR4 in prenatal brain are very hard to be found. In this study, we observed that mGluR4 was expressed in frontal lobe of prenatal human fetal brain on mRNA and protein levels. The mGluR4 mRNA increased gradually in frontal cortex from gestational 9 W to 32 W and can be detected in the VZ/SVZ where active neurogenesis took place. Consistently, the extent of immunoreactivity became stronger and the staining area expanded gradually during development. The evidence of mGluR4 expressing in prenatal human brain, especially the VZ/SVZ, makes it possible to take part in neurogenesis in human brain.

4.2. mGluR4 expressed on neural stem/progenitor cells, young neurons, mature neurons and astrocytes

In contrast to the few studies in vivo, there were many studies exploring the expression and function of mGluR4 in vitro. The first evidence was that mGluR2 and mGluR4 were detected in P19 embryocarcinoma cells that were generally regarded as an in vitro model for studying the neural differentiation [32]. Canudas also found that mGluR4 was expressed in cultured cerebellar granule cell neuroprecursors derived from 5- to 6-d-old pups, which implies mGluR4 is involved in the regulation of cerebellar development [20]. Furthermore, other studies showed mGluR4 was expressed by rodent or human NPCs in vitro. Nakamichi identified mRNA for all mGluRs except mGluR6 and mGluR7 in freshly isolated nestin-positive cortical progenitors from E15.5 mice [19]. Yoneyama found the expression of mGluR4 mRNA in freshly isolated NPCs from E18 rat [33]. Even after 12 days of neurosphere culture, the all group III mGluRs were still found on mRNA level [33]. Consistently, the study in our laboratory also found that the mRNA and protein of mGluR4 could be expressed in cultured NSCs from E15.5 rat cortex [34]. Similarly to the above in vitro results on rodents, we detected that mGluR4 was expressed by in situ neural stem/progenitor cells in human fetal cerebrum. Glutamate is one of the important extracellular factors, such as growth factors and other neurotransmitters, which exert an extrinsic regulation of neurogenesis. Its complex and delicate regulation on proliferation and differentiation of NPCs comes into play via its ionotropic receptors and metabotropic receptors [1,2,5,10]. Activity of group III mGluR subtype can significantly influence the proliferation and differentiation of NPCs based on negative regulation of adenylyl cyclase through inhibitory Gi/o proteins [10].

During development, NSCs undergo a serial of strictly regulated proliferation, differentiation and maturation; finally generate three main kinds of cells in the nervous system. In order to clarify the cell localization of mGluR4, besides neural stem/progenitor cells, we further performed the double-labeling immunofluorescence and found that mGluR4 was expressed by other types of cell such as young neurons, astrocytes and mature neurons in human fetal cerebrum. Our results are consistent with some studies that indicate mGluR4 can be expressed in mature neurons or astrocytes in adult. It is well known that mGluR4 is mainly concentrated in the presynaptic active zone of most synapses, occasionally on the postsynaptic zone [31,35–37]. In addition, mGluR4 is also expressed in other kinds of cells such as astrocytes, microglia and immature oligodendrocytes in vivo or in vitro [38–40]. The expression of mGluR4 in astrocytes and microglia is related to the neuroinflammatory CNS disorders, and mediate important neuroprotective and anti-inflammatory effects. Activation of mGluR4 negatively modulates the production of RANTES in glial cells excited by tumor necrosis factor-alpha and interferon-gamma [38]. Similarly, the activation of microglial mGluR4 may modulate the production of stable neurotoxins from the microglia in neuroinflammatory or neurodegenerative diseases [39]. However, no other report except our study here indicates the expression of mGluR4 in young neurons so that more work is still needed to prove it.

To date, limited studies concerning the mechanism of mGluR4 in neurogenesis imply two possible pathways are involved in. The first is the protection role of mGluR4 on neural stem/progenitor cells or neurons. The study in our laboratory found that agonist of mGluR4 retarded the oxidative stress-induced NSCs

death and apoptotic-associated protein activities through inhibiting the JNK and p38 pathways in vitro [34]. In addition, activation of mGluR4 also results in neuroprotection against excitotoxic insults in Parkinson's disease, ischemic stroke, as well as other CNS disorders and therefore mGluR4 will be an exciting target for the development of novel pharmacological therapies for those CNS disorders [41–45]. The second is that mGluR4 may directly regulate the process of neurogenesis in spite of the existing of conflicting results. Treatment with the agonist of mGluR4 promoted the proliferation and differentiation of NPCs accompanying changes in phosphorylation of MAPK signaling pathways [22]. However, the studies in other laboratories found that mGluR4 decreased the cell proliferation and increased the astrocytic differentiation of NPCs through a pathway related to cAMP formation [19,20]. Those inconsistent results about the role of mGluR4 on neurogenesis may need more and detailed studies to clarify.

In conclusion, the present data demonstrate that mGluR4, like the mGluR5 reported by our laboratory [13], can be expressed in the human prenatal brain on mRNA and protein levels and becomes stronger gradually during development. Moreover, the cellular localization of mGluR4 includes neural stem/progenitor cells, young neurons, astrocytes and mature neurons. This study provides important morphological evidence that mGluR4 is expressed in prenatal human cerebrum, and main kinds of cells related to neurogenesis are involved in its expression. However, because of the less attraction and inconsistent results about mGluR4 on neurogenesis, more experiments, especially in function and mechanism for mGluR4, would be conducted to corroborate the anatomical observations in this study.

5. Grant information

This work was partly supported by grants from Scientific Research Program Funded by Department of Science and Technology of Shaanxi Province (No. 2018JM7066, 2014JM4096), the Leading Disciplines Development Government Foundation of Shaanxi Province (No. [2014]3-1001).

6. Compliance with ethical standards

6.1. Conflict of interest statement

The authors declare that they have no conflicts of interest in association with this study.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee of Xian Jiaotong University Health Science Center (No. 2012-225) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Acknowledgements

This work was partly supported by grants from Scientific Research Program Funded by Department of Science and Technology of Shaanxi Province (No. 2018JM7066 by Junfeng Zhang, 2014JM4096 by Pengbo Yang), the Leading Disciplines Development Government Foundation of Shaanxi Province (No. [2014]3-1001 by Junfeng Zhang).

References

[1] Luján R, Shigemoto R, López-Bendito G. Glutamate and GABA receptor signalling in the developing brain. Neuroscience 2005;130:567–80.
[2] Jansson LC, Åkerman KE. The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells. J Neural Transm (Vienna) 2014;21:819–36.
[3] LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 1995;15:1287–98.
[4] Gould E, Cameron HA, McEwen BS. Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus. J Comp Neurol 1994;340:551–65.
[5] Cameron HA, McEwen BS, Gould E. Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci 1995;15:4687–92.
[6] Pin JP, Duvoisin R. The metabotropic glutamate receptors: structure and functions. Neuropharmacology 1995;34:1–26.
[7] Conn PJ, Pin JP. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 1997;37:205–37.
[8] Pin JP, Acher F. The metabotropic glutamate receptors: structure, activation mechanism and pharmacology. Curr Drug Targets CNS Neurol Disord 2002;1:297–317.
[9] López-Bendito G, Shigemoto R, Fairén A, Luján R. Differential distribution of group I metabotropic glutamate receptors during rat cortical development. Cereb Cortex 2002;12:625–38.
[10] Nakamichi N, Takarada T, Yoneda Y. Neurogenesis mediated by gamma-aminobutyric acid and glutamate signaling. J Pharmacol Sci 2009;110:133–49.
[11] Di Giorgi Gerevini VD, Caruso A, Cappuccio I, Ricci Vitiani L, Romeo S, Della Rocca C, et al. The mGlu5 metabotropic glutamate receptor is expressed in zones of active neurogenesis of the embryonic and postnatal brain. Brain Res Dev Brain Res 2004;150:17–22.
[12] Boer K, Encha-Razavi F, Sinico M, Aronica E. Differential distribution of group I metabotropic glutamate receptor in developing human cortex. Brain Res 2010;1324:24–33.

[13] Yang P, Zhang J, Zhao L, Jiao Q, Jin H, Xiao X, et al. Developmental distribution pattern of metabotropic glutamate receptor 5 in prenatal human hippocampus. Neurosci Bull 2012;28:704–14.
[14] Castiglione M, Calafiore M, Costa L, Sortino MA, Nicoletti F, Copani A. Group I metabotropic glutamate receptors control proliferation, survival and differentiation of cultured neural progenitor cells isolated from the subventricular zone of adult mice. Neuropharmacology 2008;55:560–7.
[15] Erichsen JL, Blaabjerg M, Bogetofte H, Serrano AM, Meyer M. Group I metabotropic glutamate receptors: a potential target for regulation of proliferation and differentiation of an immortalized human neural stem cell line. Basic Clin Pharmacol Toxicol 2015;116:329–36.
[16] Xu X, Zhang J, Chen X, Liu J, Lu H, Yang P, et al. The increased expression of metabotropic glutamate receptor 5 in subventricular zone neural progenitor cells and enhanced neurogenesis in a rat model of intracerebral hemorrhage. Neuroscience 2012;202:474–83.
[17] Brazel CY, Nuñez JL, Yang Z, Levison SW. Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. Neuroscience 2005;131:55–65.
[18] Di Giorgi-Gerevini V, Melchiorri D, Battaglia G, Ricci-Vitiani L, Ciceroni C, Busceti CL, et al. Endogenous activation of metabotropic glutamate receptors supports the proliferation and survival of neural progenitor cells. Cell Death Differ 2005;12:1124–33.
[19] Nakamichi N, Yoshida K, Ishioka Y, Makanga JO, Fukui M, Yoneyama M, et al. Group III metabotropic glutamate receptor activation suppresses self-replication of undifferentiated neocortical progenitor cells. J Neurochem 2008;105:1996–2012.
[20] Canudas AM, Di Giorgi-Gerevini V, Iacovelli L, Nano G, D'Onofrio M, Arcella A, et al. PHCCC, a specific enhancer of type 4 metabotropic glutamate receptors, reduces proliferation and promotes differentiation of cerebellar granule cell neuroprecursors. J Neurosci 2004;24:10343–52.
[21] Tian Y, Liu Y, Chen X, Kang Q, Zhang J, Shi Q, et al. AMN082 promotes the proliferation and differentiation of neural progenitor cells with influence on phosphorylation of MAPK signaling pathways. Neurochem Int 2010;57:8–15.
[22] Duan Z, Zhang X, Zhu GX, Gao Y. Xue X. Activation of mGluR4 promotes proliferation of rat neural progenitor cells while mediating activation of ERK1/2 signaling pathway. Cell Mol Biol (Noisy-le-grand) 2013;59 Suppl:OL1809-17.
[23] Cappuccio I, Verani R, Spinsanti P, Niccolini C, Gradini R, Costantino S, et al. Context-dependent regulation of embryonic stem cell differentiation by mGlu4 metabotropic glutamate receptors. Neuropharmacology 2006;51:606–11.
[24] Saxe JP, Wu H, Kelly TK, Phelps ME, Sun YE, Kornblum HI, et al. A phenotypic small-molecule screen identifies an orphan ligand-receptor pair that regulates neural stem cell differentiation. Chem Biol 2007;14:1019–30.
[25] Yang P, Zhang J, Shi H, Zhang J, Xu X, Xiao X, et al. Developmental profile of neurogenesis in prenatal human hippocampus: an immunohistochemical study. Int J Dev Neurosci 2014;38:1–9.
[26] Schlett K. Glutamate as a modulator of embryonic and adult neurogenesis. Curr Top Med Chem 2006;6:949–60.
[27] Catania MV, Landwehrmeyer GB, Testa CM, Standaert DG, Penney Jr JB, Young AB. Metabotropic glutamate receptors are differentially regulated during development. Neuroscience 1994;61:481–95.
[28] Makoff A, Lelchuk R, Oxer M, Harrington K, Emson P. Molecular characterization and localization of human metabotropic glutamate receptor type 4. Brain Res Mol Brain Res 1996;37:239–48.
[29] Phillips T, Makoff A, Brown S, Rees S, Emson P. Localization of mGluR4 protein in the rat cerebral cortex and hippocampus. NeuroReport 1997;8:3349–54.
[30] Elezgarai I, Benítez R, Mateos JM, Lázaro E, Osorio A, Azkue JJ, et al. Developmental expression of the group III metabotropic glutamate receptor mGluR4a in the medial nucleus of the trapezoid body of the rat. J Comp Neurol 1999;411:431–40.
[31] Corti C, Aldegheri L, Somogyi P, Ferraguti F. Distribution and synaptic localization of the metabotropic glutamate receptor 4 (mGluR4) in the rodent CNS. Neuroscience 2002;110:403–20.
[32] Heck S, Enz R, Richter-Landsberg C, Blohm DH. Expression of eight metabotropic glutamate receptor subtypes during neuronal differentiation of P19 embryocarcinoma cells: a study by RT-PCR and in situ hybridization. Brain Re. Dev Brain Res 1997;101:85–91.
[33] Yoneyama M, Nakamichi N, Fukui M, Kitayama T, Georgiev DD, Makanga JO, et al. Promotion of neuronal differentiation through activation of Nmethyl- D-aspartate receptors transiently expressed by undifferentiated neural progenitor cells in fetal rat cortex. J Neurosci Res 2008;86:2394–402.
[34] Zhang Z, Ma W, Wang L, Gong H, Tian Y, Zhang J, et al. Activation of type 4 metabotropic glutamate receptor attenuates oxidative stress-induced death of neural stem cells with inhibition of JNK and p38 MAPK signaling. Stem Cells Dev 2015;24:2709–22.
[35] Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, et al. Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 1996;381:523–5.
[36] Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, et al. Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 1997;17:7503–22.
[37] Kinoshita A, Shigemoto R, Ohishi H, van der Putten H, Mizuno N. Immunohistochemical localization of metabotropic glutamate receptors, mGluR7a and mGluR7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. J Comp Neurol 1998;393:332–52.
[38] Besong G, Battaglia G, D'Onofrio M, Di Marco R, Ngomba RT, Storto M, et al. Activation of group III metabotropic glutamate receptors inhibits the production of RANTES in glial cell cultures. J Neurosci 2002;22:5403–11.
[39] Taylor DL, Diemel LT, Pocock JM. Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. J Neurosci 2003;23:2150–60.
[40] Spampinato SF, Merlo S, Chisari M, Nicoletti F, Sortino MA. Glial metabotropic glutamate receptor-4 increases maturation and survival of oligodendrocytes. Front Cell Neurosci 2015;8:462.
[41] Johnson KA, Conn PJ, Niswender CM. Glutamate receptors as therapeutic targets for Parkinson's disease. CNS Neurol Disord Drug Targets 2009;8:475–91.
[42] Moyanova SG, Mastroiacovo F, Kortenska LV, Mitreva RG, Fardone E, Santolini I, et al. Protective role for type 4 metabotropic glutamate receptors against ischemic brain damage. J Cereb Blood Flow Metab 2011;31:1107–18.
[43] Caraci F, Battaglia G, Sortino MA, Spampinato S, Molinaro G, Copani A, et al. Metabotropic glutamate receptors in neurodegeneration/neuroprotection: still a hot topic? Neurochem Int 2012;61:559–65.
[44] Nicoletti F, Bruno V, Ngomba RT, Gradini R, Battaglia G. Metabotropic glutamate receptors as drug targets: what's new? Curr Opin Pharmacol 2015;20:89–94.
[45] Domin H, Przykaza Ł, Jantas D, Kozniewska E, Boguszewski PM, Śmiałowska M. Neuroprotective potential of the group III mGlu receptor agonist ACPT-I in animal models of ischemic stroke: In vitro and in vivo studies. Neuropharmacology 2016;102:276–94.